

Taxonomic Study of *Bacillus coagulans* Hammer 1915 with a Proposal for *Bacillus smithii* sp. nov.

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Guanine-plus-cytosine (G+C) content determinations, deoxyribonucleic acid (DNA) relatedness estimations, and phenotypic similarity analyses of 90 strains identified previously as *Bacillus coagulans* Hammer 1915 revealed that 52 (group 1) strains were *Bacillus coagulans* sensu stricto; 30 of the remaining organisms segregated into two distinct DNA relatedness groups, one consisting of 26 (group 2) strains and the other of 4 (group 4) organisms. Five (group 3) strains were *Bacillus licheniformis*, and three (group 5) strains were *B. stearothermophilus*. Because group 2 was a major cluster of strains which did not correspond in their characteristics to any previously known group, efforts were made to determine its taxonomic position. Group 2, with G+C contents ranging from 37 to 40 mol%, was compared with other species generally characterized as having G+C contents ranging from 37 to 44 mol% or capable of growing at 50°C or both (namely, *Bacillus alcalophilus*, *Bacillus azotoformans*, *Bacillus badius*, *Bacillus firmus*, *Bacillus globiformis*, *Bacillus laterosporus*, *Bacillus macquariensis*, *Bacillus marinus*, *Bacillus megaterium*, *Bacillus pumilus*, and *Bacillus subtilis*). Low DNA relatedness values and poor matching of phenotypic characteristics strongly indicated that group 2 organisms are strains of a new species, for which the name *Bacillus smithii* is proposed. The type strain of the new species is strain NRRL NRS-173.

Bacillus coagulans is an economically important species because it is frequently involved in the coagulation of canned milk and flat-souring of other carbohydrate-containing canned foods (17). The production of high concentrations of L-(+)-lactic acid causes the spoilage (40). Hammer first isolated this thermophilic organism from spoiled canned milk and described it as a new species in 1915 (22). In ensuing studies, morphological and physiological inconsistencies exhibited by the species have perplexed taxonomists. For example, Smith et al. (45), Bradley and Franklin (5), and Seki et al. (43) observed that the morphologies of the cells, spore surfaces, and sporangia could vary from strain to strain. Based on similarity analyses of API tests, Logan and Berkeley (26) noted that many *B. coagulans* strains clustered outside the *B. coagulans* phenon. Wolf and Barker (51) and Klaushofer and Hollaus (23) identified two types based on differences in maximum growth temperatures and in some physiological and biochemical characteristics, such as growth at pH 4.5 and 7.7, growth at 60°C, production of acetylmethylcarbinol, coagulation of litmus milk, and fermentation of arabinose, mannitol, starch, and sucrose. In contrast, strains studied by Gordon and Smith (19) did not separate so neatly into two distinct groups. Moreover, Priest et al. (38) demonstrated an overall homogeneity of *B. coagulans* by performing a numerical analysis of data published by Gordon et al. (18). The high variability has undoubtedly encouraged the creation of subjective synonyms, such as "*Bacillus thermoacidurans*" (3), "*Bacillus dextralacticus*" (1), "*Bacillus thermoacidificans*" (39), and "*Lactobacillus cereale*" (35).

The reported wide range of 41 to 55 mol% for the guanine-plus-cytosine (G+C) contents of deoxyribonucleic acids (DNAs) (6, 30, 37) indicated genetic heterogeneity in *B. coagulans*. Blumenstock (Ph.D. thesis, University of Gottingen, Gottingen, Federal Republic of Germany, 1984)

found two groups of *B. coagulans* strains with different phenotypic and genotypic characteristics. Finding the level of DNA relatedness among *B. coagulans* strains to be high, other workers considered the species to be genetically homogeneous (37, 43). The genetic homogeneity and phenotypic homogeneity reported by some workers probably reflect the fortuitous selection of strains which happen to be closely related.

The variability of *B. coagulans* appears in part to be a true characteristic of the species; also, it probably reflects the genetic heterogeneity of the taxon. To determine the degree to which the phenotypic heterogeneity could be attributed to genetic heterogeneity and to phenotypic variation, in this study we assessed the extent of genetic homogeneity of the species by measuring G+C contents and levels of DNA relatedness.

MATERIALS AND METHODS

Bacterial strains. The *B. coagulans* strains used in this study are listed in Table 1. Also used in this study were *Bacillus alcalophilus* Vedder 1934 NRRL B-14309^T (= DSM 485^T) (T = type strain), *Bacillus alvei* Cheshire and Cheyne 1885 NRRL B-383^T (= DSM 29^T), *Bacillus azotoformans* Pichinoty, de Barjac, Mandel, and Asselineau 1983 NRRL B-14310^T (= DSM 1046^T), *Bacillus badius* Batchelor 1919 NRRL NRS-663^T (= DSM 23^T), *Bacillus brevis* Migula 1900 NRRL NRS-604^T (= DSM 30^T), *Bacillus firmus* Bredemann and Werner 1933 NRRL B-14307^T (= DSM 12^T), *Bacillus globisporus* Larkin and Stokes 1967 NRRL B-3396^T (= DSM 4^T), *Bacillus laterosporus* Laubach 1916b NRRL NRS-314^T (= DSM 25^T), *Bacillus licheniformis* (Weigmann) Chester 1901 NRRL NRS-1264^T (= DSM 13^T), *Bacillus macquariensis* Marshall and Ohye 1966 NRRL B-14306^T (= DSM 2^T), *Bacillus marinus* (Ruger and Richter 1979) Ruger 1983 NRRL B-14321^T (= DSM 1297^T), *Bacillus megaterium* de Bary 1884 NRRL B-14308^T (= DSM 32^T), *Bacillus polymyxa* (Prazmowski) Mace 1889 NRRL NRS-1105^T (= DSM 36^T),

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TABLE 1. List of *B. coagulans* strains used in this study

Laboratory no.	Received as strain:	Source ^a	Strain history ^b
NRRL B-768	NRS-784	1	C. H. Werkman, " <i>Bacillus dextralacticus</i> "; (DSM 2311) ^c
NRRL B-1103	ATCC 8038	2	NCA 43P, " <i>B. thermoacidurans</i> "
NRRL B-1167	NRS-13	1	H. C. Curran 195
NRRL B-1168	NRS-14	1	H. C. Curran 1460
NRRL B-1175	NRS-21	1	R. W. Pilcher 94S, " <i>B. thermoacidurans</i> "
NRRL B-1178	NRS-24	1	R. W. Pilcher 4E, " <i>B. thermoacidurans</i> "
NRRL B-1179	NRS-25	1	R. W. Pilcher 6, " <i>B. thermoacidurans</i> "
NRRL B-1180	NRS-26	1	R. W. Pilcher 43P, " <i>B. thermoacidurans</i> "
NRRL B-1181	NRS-27	1	R. W. Pilcher 78G, " <i>B. thermoacidurans</i> "; (DSM 2383)
NRRL B-4284	8173	3	From raw buffalo milk
NRRL B-14027	212	3	From raw buffalo milk
NRRL B-14311	DSM 459	4	F. Hollaus E28-66, from sugar beet juice
NRRL B-14312	DSM 460	4	F. Hollaus E30-66, from sugar beet juice
NRRL B-14313	DSM 2319	4	F. Hollaus E35-66, from sugar beet juice
NRRL B-14314	DSM 2320	4	F. Hollaus E45-66, from sugar beet juice
NRRL B-14315	DSM 2321	4	F. Hollaus E50-67, from sugar beet juice
NRRL B-14316	DSM 2313	4	NCIB 10278 from R.S.C. Aytoun 186
NRRL B-14317	DSM 2357	4	NCIB 10279 from R.S.C. Aytoun a-1
NRRL B-14318	DSM 2358	4	NCIB 10280 from R.S.C. Aytoun a-7
NRRL NRS-54 to -58	NRS-54 to -58	5	NCA 4167, NCA F43, NCA 52-240, NCA 4578, NCA 4110
NRRL NRS-83	NRS-83	5	E. McCoy, Pan strains E
NRRL NRS-96 to -105	NRS-96 to -105	5	N. R. Smith, from cream
NRRL NRS-114, -115, -121, -126, -134, -138 to -140, -142 to -145	NRS-114, -115, -121, -126, -134, -138 to -140, -142 to -145	5	NCA 1215, NCA 1264, NCA 1460, NCA 1734, NCA 848, NCA 518, NCA 698, NCA 880, NCA 1500, NCA 11878, NCA 2, NCA 4
NRRL NRS-169 to -176	NRS-169 to -176	5	N. R. Smith, from cheese
NRRL NRS-177 to -184	NRS-177 to -184	5	N. R. Smith, from alfalfa silage
NRRL NRS-185	NRS-185	5	E. J. Hehre N9
NRRL NRS-186	NRS-186	5	P. Renco, " <i>B. thermoacidurans</i> "
NRRL NRS-190a, -191, -192a	NRS-190 to -192	5	N. R. Smith, from cheese
NRRL NRS-198, -200	NRS-198, -200	5	NCA C-1655, NCA C-2291, NCA C-1657
NRRL NRS-609 ^T	NRS-609 ^T	5	J. R. Porter from B. W. Hammer; (DSM 1 ^T , ATCC 7050 ^T); type strain
NRRL NRS-770	NRS-770	5	NCA, " <i>B. thermoacidurans</i> "
NRRL NRS-795 to -797	NRS-795 to -797	5	B. W. Hammer 195, 196, 198, from canned evaporated milk
NRRL NRS-798	NRS-798	5	B. W. Hammer 200; from canned evaporated milk; (DSM 2356)
NRRL NRS-905	NRS-905	5	J. R. Porter from M. Schieblich, " <i>Bacillus modestus</i> " M21
NRRL NRS-1371, -1375, -1376	NRS-1371, -1375, -1376	5	W. G. Walters 71, 5-2, 5-3; from hot spring
NRRL NRS-2006 to -2008	NRS-2006 to -2008	5	R. F. Brooks, " <i>Bacillus calidolactis</i> "
NRRL NRS-2011	NRS-2011	5	E. Olsen, " <i>B. thermoacidurans</i> ," from sugar-refining laboratory
NRRL NRS-2012	NRS-2012	5	E. Olsen, " <i>L. cereale</i> " U, from sugar-refining laboratory; (DSM 2350)
NRRL NRS-2013	NRS-2013	5	E. Olsen, " <i>L. cereale</i> " B, from sugar-refining laboratory
NRRL NRS-2016	NRS-2016	5	C. S. Pederson, from spoiled canned tomatoes
NRRL NRS-2020	NRS-2020	5	C. S. Pederson, <i>Lactobacillus delbrueckii</i> 1260
NRRL NRS-2021 to -2023	NRS-2021 to -2023	5	NCA 3991, NCA 1035, NCA 1036; " <i>B. thermoacidurans</i> "

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^b NCA, National Canners Association, San Francisco, Calif.

^c Names in quotation marks are not on the Approved Lists of Bacterial Names (44) and have not been validly published since January 1980. Designations in parentheses are equivalent strain numbers.

Bacillus pumilus Meyer and Gottheil 1901 NRRL NRS-272^T (= DSM 27^T), *Bacillus stearothermophilus* Donk 1920 NRRL B-1172^T (= DSM 22^T), and *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872 NRRL NRS-744^T (= DSM 10^T). These strains are maintained by the Agricultural Research Service Culture Collection (NRRL) at the Northern Regional Research Center, Peoria, Ill., and by the Deutsche Sammlung von Mikroorganismen (DSM), Gottingen, Federal Republic of Germany. The NRRL strain designations include the prefixes B- and NRS-; the prefix B- denotes strains that were obtained directly from a source or strains that were isolated at the Northern Regional Research Center, and the prefix NRS- designates strains of the

Bacillus collection of N. R. Smith, which has been deposited in toto at the Northern Regional Research Center by R. E. Gordon.

For maintenance all strains were grown on nutrient agar containing 5 mg of MnSO₄ per liter or soil extract agar (18) until spores were formed. *B. coagulans* was grown at 45°C, *B. stearothermophilus* was grown at 50°C, *B. globisporus*, *B. macquariensis*, and *B. marinus* were grown at 25°C, and all other strains were grown at 30°C. The cultures were stored at 4°C and were transferred semiannually.

DNA investigations. The cells were grown in nutrient broth under agitation and were harvested by centrifugation in the

late logarithmic growth phase. Previous publications have described the procedure for preparing highly purified DNA samples by hydroxyapatite chromatography (34) and the methods used for estimating the extent of DNA reassociation by spectrophotometric determination of renaturation rates with a Gilford model 2600 ultraviolet spectrophotometer equipped with a model 2527 thermoprogrammer (8, 34). DNA relatedness values were calculated by using the equation of De Ley (14).

The buoyant densities of DNA samples were measured by CsCl density gradient centrifugation in a Beckman model E ultracentrifuge to determine G+C contents (42). *Micrococcus luteus* (synonym, "*Micrococcus lysodeikticus*") DNA with a buoyant density of 1.724 g/cm³ (49), which was purchased from Sigma Chemical Co., St. Louis, Mo., served as an internal standard.

The G+C contents of strains NRRL B-14311, NRRL B-14312, NRRL B-14313, NRRL B-14314, NRRL B-14315, NRRL B-14316, NRRL B-14317, and NRRL B-14318 were calculated from the thermal melting point (T_m) by using the following equation: G+C content = $2.44 \times (T_m - 69.4)$ (13). All values were corrected for the differences between the determined G+C content of the reference *Escherichia coli* DNA (Sigma) and 50.9 mol%, the value used by De Ley for *E. coli*. A detailed description of the method has been given by Mandel and Marmur (28) and Fahmy et al. (16). The DNAs used for these determinations were purified by a modification of the method of Marmur (16, 29).

Characterization. Morphological properties were examined by phase-contrast microscopy, using slides coated with a thin layer (1 mm) of purified agar (catalog no. 1613; Merck & Co., Inc., Rahway, N.J.). The sizes of cells were calculated from photomicrographs (Photomicroscope II; Zeiss). The mode of flagellation was examined by using the staining method described by Mayfield and Inniss (31) or Kodaka et al. (24). To determine the Gram reaction, we used the method of Bartholomew (2), with *n*-propanol as the decolorizing agent. The Gram reaction was compared with lysis of cells by 3% (wt/vol) KOH (20) and the Cerny aminopeptidase test (7). The maximum growth temperature was tested in a thermostatically controlled water bath in 5°C intervals. The cultures were inoculated onto nutrient agar slants.

Unless stated otherwise, the methods of Gordon et al. (18) were used to characterize the strains. The carbohydrate fermentation tests were conducted in broth and not on agar slants. To a basal medium modified to contain (per liter) 1 g of (NH₄)₂HPO₄, 0.7 g of yeast extract, 0.2 g of KCl, 0.2 g of MgSO₄ · 7H₂O, and 15 ml of a 0.04% solution of bromocresol purple was added a separately sterilized solution of one of the following sugars to a final concentration of 1%: L-arabinose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, melibiose, L-rhamnose, D-ribose, salicin, D-sorbitol, sucrose, trehalose, and D-xylose. The organic acid utilization tests included acetate, fumarate, malate, and succinate tests in addition to citrate and propionate tests. For these utilization tests, the basal medium was modified to contain (per liter) 0.5 g of yeast extract, 0.4 g of MgSO₄ · 7H₂O, and 0.03 g of bromthymol blue. Medium containing no organic acids was used as a control. Arginine, lysine, and ornithine decomposition was determined in Moeller decarboxylase broth (32). Hydrogen sulfide production was detected by stab culturing in triple sugar iron agar (21). Urease activity was determined by the method of Edwards and Ewing (15). Acetylmethylcarbinol was detected with reagents described by Coblenz (9). Hydrolysis of pullulan was determined by the methods of Morgan et al.

(33). The oxidase reaction and hydrolysis of chitin and DNA were determined by the method of Cowan (11). Diaminopimelic acid in the vegetative cell walls was determined qualitatively by the thin-layer chromatographic method of Kutzner (25). The qualitative menaquinone contents of vegetative cells were determined by the method of Collins et al. (10).

In addition to the standard methods used in *Bacillus* diagnostics, the API 20E and API 50CHB test systems were used according to the instructions of the manufacturer.

Numerical analyses. The organisms were screened for 49 differential characteristics, and positive and negative results were coded as 1 and 0, respectively. Similarity among strains was estimated by means of the simple matching coefficient, and clustering was based on the unweighted pair group arithmetic average algorithm (46, 47). Computations were carried out with an IBM PC computer by using the TAXO-NOPC program of D. Labeda (Northern Regional Research Center).

RESULTS

Based on the analyses of G+C contents shown in Table 2, the 90 *B. coagulans* strains separated into three clusters. The 57 strains in the largest cluster (groups 1 and 3) had G+C contents ranging from 45 to 47 mol%, a range that included the base content (45 mol%) of type strain NRRL NRS-609. Of the 33 remaining organisms, 30 had G+C contents which ranged from 37 to 40 mol% (groups 2 and 4), and 3 had G+C contents which ranged from 52 to 53 mol% (group 5).

As indicated by the data in Table 2, the strains studied could also be separated in five distinct DNA relatedness groups. Group 1 included 52 strains with G+C contents ranging from 45 to 47 mol%, and these strains gave high DNA complementarity values of 76 to 100% with *B. coagulans* type strain NRRL NRS-609 (= DSM 1). As shown in Table 3, DNA relatedness values of 16 to 34% were measured between the group 1 reference strain and the following strains of currently recognized species: *B. alvei* NRRL B-383^T, *B.adius* NRRL NRS-633^T, *B. brevis* NRRL NRS-604^T, *B. licheniformis* NRRL NRS-1264^T, *B. macerans* NRRL B-4267^T, *B. polymyxa* NRRL NRS-1105^T, *B. stearothermophilus* NRRL B-1172^T, and *B. subtilis* NRRL NRS-744^T. These currently recognized species were selected because they grow at 50 to 60°C or have G+C contents of 43 to 52 mol% or both.

Group 3 organisms had G+C contents of 45 to 46 mol%. However, low DNA reassociation values of 18 to 31% measured with the group 1 reference strain indicated that the group 3 organisms were not closely related genetically to group 1 (Table 2). Group 3 consisted of reference strain NRRL B-4284 and four other strains with which high DNA relatedness values (99 to 100%) were obtained. *B. licheniformis* NRRL NRS-1264^T and reference strain NRRL B-4284 were closely related genetically, as suggested by the 100% relatedness of their DNAs (Table 3). High DNA relatedness values of 95 to 100% (data not shown) were also observed between strain NRRL NRS-1264^T and other group 3 strains.

Included in groups 2 and 4 were strains with G+C contents of 37 to 40 mol% (Table 2). Although selected strains yielded low DNA complementarity values of 14 to 37% with group 1, 3, 4, and 5 reference strains, group 2 strains (26 strains) gave high DNA relatedness values of 78 to 100% with reference strain NRRL NRS-173. Group 4 consisted of reference strain NRRL NRS-139 and three genetically

TABLE 2. DNA relatedness of *B. coagulans* strains

NRRL strain no.	G+C content (mol%)	% Reassociation with DNA from strain: ^a				
		NRRL NRS-609 ^T	NRRL NRS-173	NRRL B-4284	NRRL NRS-139	NRRL B-14317
Group 1						
B-768	45	98				38
B-1103	45	96		28		
B-1167	45	92	31			
B-1168	45	93	28	34		37
B-1178	45	91			33	
B-1179	45	87				
B-1180	45	100			29	27
B-1181	45	93	33	29		
NRS-54	45	91	29	36		37
NRS-55	45	100			33	
NRS-56	46	95				
NRS-57	45	94				
NRS-58	46	97				
NRS-83	46	82			32	39
NRS-105	45	92	30	27		
NRS-114	47	100			35	
NRS-115	45	87				
NRS-121	45	94		27		
NRS-126	45	100				
NRS-134	45	95				
NRS-144	45	95	30			
NRS-145	46	88			29	
NRS-171	46	80	33		34	
NRS-172	45	85		27		
NRS-175	45	80			28	
NRS-176	45	88				
NRS-177	47	91				28
NRS-178	45	83				
NRS-181	45	76	28			39
NRS-182	45	80		29		
NRS-183	46	100				
NRS-184	46	95			34	
NRS-186	45	90				
NRS-190a	47	85	27			
NRS-191	45	96		27		
NRS-192a	45	91			30	
NRS-198	45	100				
NRS-609 ^T	45	(100) ^b	30			
NRS-770	45	80				33
NRS-795	45	100				
NRS-796	45	100				
NRS-797	46	82				
NRS-798	45	95		30		
NRS-2007	45	100			34	
NRS-2011	45	87	29			
NRS-2012	45	95				
NRS-2013	45	100				
NRS-2016	45	80	33			
NRS-2020	46	78				30
NRS-2021	45	100				
NRS-2022	45	99				
NRS-2023	46	100				
Group 2						
B-1175	40	23	100	21	30	32
B-14311	39	32	87			
B-14312	40	31	95			
B-14313	39	30	89			
B-14314	40	29	90			
B-14315	39	32	98			
NRS-96	40	14	100		29	
NRS-97	40	28	100			
NRS-98	40	23	94			26
NRS-99	39	25	99		28	
NRS-100	39	23	99	21		
NRS-101	39	33	96	22		32
NRS-102	39	33	86	25	28	
NRS-103	40	34	100		24	29
NRS-104	40	35	87		29	
NRS-138	40	20	98		29	37

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TABLE 2—Continued

NRRL strain no.	G+C content (mol%)	% Reassociation with DNA from strain: ^a				
		NRRL NRS-609 ^T	NRRL NRS-173	NRRL B-4284	NRRL NRS-139	NRRL B-14317
NRS-140	40	35	100	20		33
NRS-142	40	34	94	23		
NRS-143	39	29	99		28	34
NRS-169	40	34	98		30	
NRS-170	40	31	97	20		
NRS-173 ^c	40	30	(100)			28
NRS-174	40	26	100			
NRS-199	38	29	78	25		
NRS-1371	40	32	100	24		36
NRS-2008	39	29	100	22	22	36
Group 3						
B-4284 ^c	46	18	22	(100)	23	29
B-14027	46	23	10	100	22	30
NRS-185	45	30	18	99	27	29
NRS-1375	46	30	20	100	23	35
NRS-1376	45	31	13	100	23	38
Group 4						
NRS-139 ^c	38	26	20	13	(100)	36
NRS-200	37	38	24	15	99	35
NRS-905	37	32	23	25	99	30
NRS-2006	37	29	27	18	99	29
Group 5						
B-14316	52	33	33	29	36	98
B-14317 ^c	52	31	35	30	32	(100)
B-14318	53	29	34	30	27	84

^a Reassociation values are averages of two determinations; the maximum difference noted between determinations was 8%.

^b Values in parentheses indicate that, by definition, the reassociation value was 100%.

^c Reference strain for the group.

closely related strains (DNA relatedness values, 99%). Low DNA relatedness values of 20 to 34% were measured between group 2 and 4 reference strains and the type strains of the currently recognized species (Table 3).

Group 5 included three strains that have G+C contents of 52 to 53 mol% and give high DNA complementarity values with reference strain NRRL B-14317. Low levels of DNA relatedness to their reference strains (27 to 36%) indicate that group 5 is not closely related genetically to group 1, 2, 3, or 4. The high level of DNA relatedness of *B. stearothermo-*

philus NRRL B-1172^T and NRRL B-14317 suggests that group 5 must be *B. stearothermophilus* (Table 3).

Figure 1 is a dendrogram, based on phenotypic characters and plotted from computer-generated linkage data, that shows the clustering of strains by using a simple matching coefficient and an unweighted average-linkage algorithm. At a similarity level of 85%, the matrix formed five phenotypes. Significantly, the strains found in any one of the phenotypes were also the strains collected into groups on the basis of their high levels of DNA relatedness. For example, the strain

TABLE 3. DNA relatedness of group reference strains and *Bacillus* type strains

Strain	G+C content (mol%) ^a	% Reassociation with DNA from group reference strain: ^b				
		NRRL NRS-609 ^T	NRRL NRS-173	NRRL B-4284	NRRL NRS-139	NRRL B-14317
<i>B. alcalophilus</i> NRRL B-14309 ^T	37.0		33		25	
<i>B. megaterium</i> NRRL B-14308 ^T	37.1		29		29	
<i>B. marinus</i> NRRL B-14321	37.7		23		31	
<i>B. azotoformans</i> NRRL B-14310 ^T	39.0		32		24	
<i>B. macquariensis</i> NRRL B-14306 ^T	39.3		29		29	
<i>B. globisporus</i> NRRL B-3396 ^T	39.7		22		27	
<i>B. laterosporus</i> NRRL NRS-314 ^T	40.2		23		33	
<i>B. firmus</i> NRRL B-14307 ^T	41.5		34		33	
<i>B. pumilus</i> NRRL NRS-272 ^T	42.0		30		31	
<i>B. subtilis</i> NRRL NRS-744 ^T	42.9	23	25	21		
<i>B. badius</i> NRRL NRS-663 ^T	43.8	24	21	24		
<i>B. polymyxa</i> NRRL NRS-1105 ^T	44.5	32	23	28		
<i>B. alvei</i> NRRL B-383 ^T	44.6	34	21	20		
<i>B. licheniformis</i> NRRL NRS-1264 ^T	46.5	29	23	100		
<i>B. brevis</i> NRRL NRS-604 ^T	47.5	16	20	22		
<i>B. stearothermophilus</i> NRRL B-1172 ^T	51.9	28	20			85
<i>B. macerans</i> NRRL B-4267 ^T	52.2	30	25			35

^a Data from reference 16.

^b Reassociation values are averages of two determinations; the maximum difference noted between determinations was 6%.

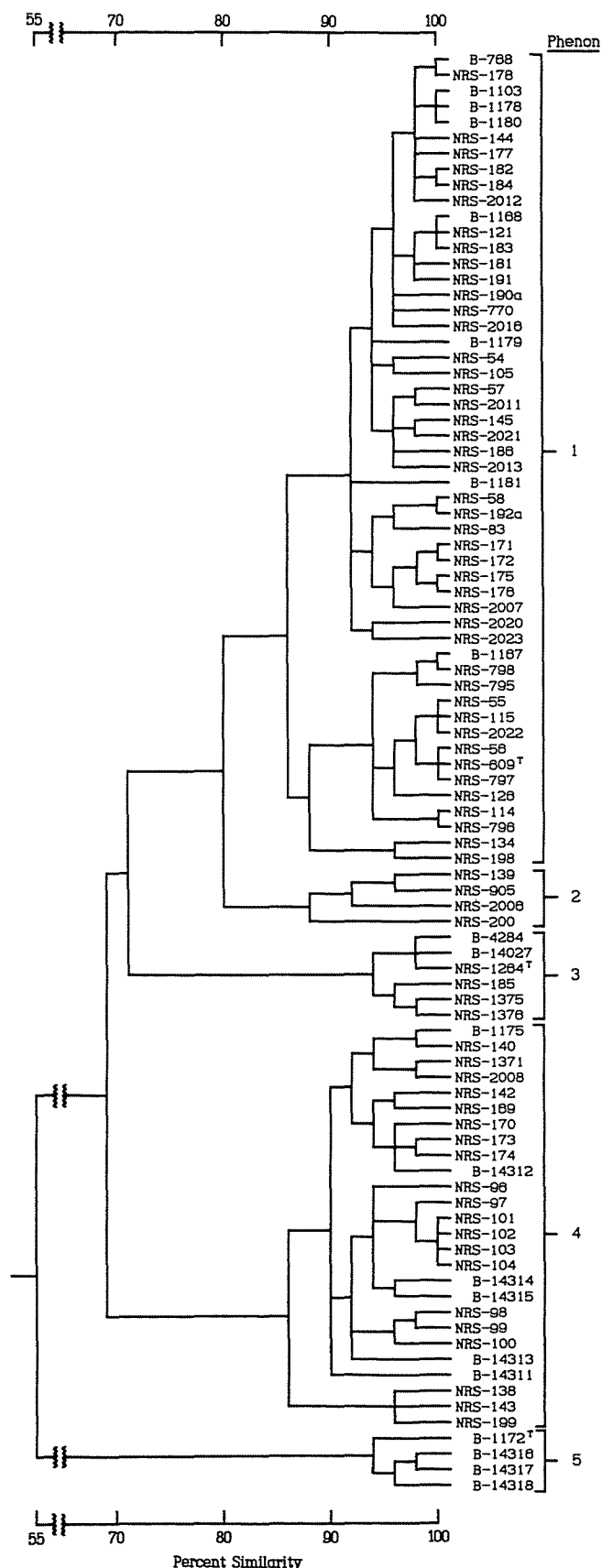


FIG. 1. Dendrogram showing relationship among NRRL strains studied, based on simple matching coefficient and unweighted average-linkage clustering.

compositions of DNA relatedness groups 1 and 2 were identical to those of phenon 1 and 4, respectively. Relatedness groups 3 and 5 clustered with *B. licheniformis* (phenon 3) and *B. stearothermophilus* (phenon 5), respectively. The salient characteristics that distinguish the five DNA relatedness groups are listed in Table 4. These data clearly demonstrated that each of the five DNA relatedness groups not only is genetically distinct but also is phenotypically distinct.

A total of 9 group 1 and 22 group 2 strains were further characterized by using the API 20E and API 50CHB systems (Table 5). The results from the API system tests and the standard tests were generally similar. Discrepancies were found in the number of strains that produce acid from glycerol and starch and that synthesize acetylmethylcarbinol. In their study of *Bacillus* spp., Logan and Berkeley (27) similarly observed the tendencies of the API 20E system to give higher numbers of positive acetylmethylcarbinol tests than the standard procedure. The use of pyruvate instead of glucose as the substrate may explain the results of the API system tests.

The API 50CHB system gave variable fermentation reactions for many of the carbohydrates. Definite differences were noted between groups 1 and 2 for the fermentation of lactose, mannitol, melibiose, *N*-acetylglucosamine, raffinose, and starch. According to the API 50CHB system, groups 1 and 2 did not ferment adonitol, *L*-arabitol, dulcitol, *D*- and *L*-fucose, inulin, 2- and 5-ketogluconate, *D*-lyxose, melezitose, α -methyl-*D*-mannoside, β -methyl-xyloside, *L*-sorbitose, *D*-tagatose, and *L*-xylose.

The major groups, groups 1 and 2, closely resembled each other morphologically and shared many biochemical and physiological characteristics. Group 1 strains grown on nutrient agar had cell widths of 0.4 to 0.8 μ m, and group 2 strains grown on the same medium had cell widths of 0.6 to 1.0 μ m. Both groups were peritrichously flagellated. On nutrient agar supplemented with $MnSO_4$, they produced terminally or subterminally located oval or cylindrical spores that measured 0.6 to 0.8 by 1.3 to 1.7 μ m; the sporangia were slightly swollen or not swollen at all.

Young cells of both groups were gram positive. With increasing age the cells became gram variable and finally gram negative. The KOH and aminopeptidase tests were negative, as is typical for gram-positive organisms. Vegetative cells of all strains contained diaminopimelic acid in their cell walls.

Biochemically and physiologically, all group 1 and 2 strains formed catalase; fermented *D*-fructose, *D*-glucose, and trehalose; were facultatively anaerobic; grew at pH 5.7; and hydrolyzed DNA and hippurate. None of the strains grew in the presence of 3% NaCl or lysozyme; hydrolyzed casein, chitin, egg yolk lecithin, or gelatin; produced dihydroxyacetone, H_2S , or indole; or decomposed arginine, lysine, ornithine, phenylalanine, tyrosine, or urea.

DISCUSSION

Analyses of DNA relatedness levels and phenotypic similarity demonstrated five distinct groups among the 90 *B. coagulans* sensu lato strains which we studied. Since it includes the type strain (NRRL NRS-609), DNA relatedness group 1 represents *B. coagulans* sensu stricto. Groups 3 and 5 are *B. licheniformis* and *B. stearothermophilus*, respectively. Groups 2 and 4 are apparently not closely related genetically to any of the currently recognized species that have G+C contents in the 37- to 40-mol% range or grow at 50

TABLE 4. Salient characteristics of the *B. coagulans* DNA relatedness groups

Characteristics	Group 1 (n = 52)	Group 2 (n = 26)	Group 3 (n = 5)	Group 4 (n = 4)	Group 5 (n = 3)
Anaerobic growth	100 ^a	100	100	0	0
Growth at:					
pH 4.5 in nutrient broth	100	0	0	0	0
pH 7.7 in nutrient broth	100	0	100	100	100
pH 5.7 in Sabouraud broth	100	100	100	100	0
Growth in:					
7% NaCl	100	0	100	0	0
0.02% Sodium azide	100	5	0	0	0
Growth at:					
25°C	100	100	100	100	0
55°C	100	100	100	0	100
60°C	100	100	0	0	100
65°C	0	73	0	0	100
70°C	0	0	0	0	100
Nitrate reduction to nitrite	25	0	100	0	100
Denitrification	0	0	0	0	100
Acetylmethylcarbinol in Voges-Proskauer broth ^b	100	0	100	0	0
Oxidase reaction	5	100	0	0	0
Arginine dihydrolase	0	0	100	0	0
Utilization of:					
Citrate	33	96	100	0	0
Propionate	0	67	100	0	0
Hydrolysis of:					
Casein	0	0	100	0	100
Gelatin	0	0	100	0	100
Pullulan	100	5	100	0	100
Starch	100	21 (w)	100	0	100
Litmus milk:					
Coagulated	100	0	0	0	0
Acidified	100	0	0	0	0
Fermentation of:					
L-Arabinose	71	67	100	100	100
Cellobiose	77	0	100	75	0
D-Galactose	100	86	60	75	100
Lactose	100	0	0	0	0
Maltose	100	57	100	100	67
D-Mannitol	64	86	100	100	0
D-Mannose	100	38	100	100	100
Melibiose	100	10	40	0	100
L-Rhamnose	64	29	60	100	0
D-Ribose	67	90	100	75	100
Salicin	77	15	100	25	100
D-Sorbitol	52	38	80	50	0
Starch	100	0	100	0	100
Sucrose	100	10	100	50	100
D-Xylose	67	95	100	100	100

^a The values are percentages of strains showing positive reactions. w, Weak.

^b The pH values in Voges-Proskauer broth were 4.0 to 4.4 (group 1), 4.3 to 4.7 (group 2), 5.1 to 6.6 (group 3), 6.6 to 7.0 (group 4), and 4.2 to 5.8 (group 5).

to 60°C or both (Table 2). The data suggest that groups 2 and 4 may be strains of yet-unnamed species.

The gathering of numerous strains of five separate species within the *B. coagulans* sensu lato group partially accounts for the observation of variants or subgroups by some workers (18, 23, 51). Some of the phenotypic differences reported by Wolf and Barker (51) are noted when two major clusters (groups 1 and 2) are compared (Table 4). For example, the data reveal that *B. coagulans* sensu stricto strains (group 1) produce acetylmethylcarbinol; coagulate milk; are oxidase negative; hydrolyze starch and pullulan; frequently ferment cellobiose, lactose, and salicin; grow in the presence of 0.02% azide; and grow variably at 60°C and not at all at 65°C. Group 2 strains grow at 60°C and variably at 65°C, are oxidase positive, and are essentially negative for the other characteristics. The group 1 strains observed in this study are very similar to the type B organisms of Wolf and Barker

(51), group 2 strains correspond to the type A strains, and groups 3 through 5 may be the same as the intermediate types.

Although intermixing of species has made *B. coagulans* sensu lato a phenotypically heterogeneous taxon, the separation of species circumscribed on the basis of DNA relatedness has not necessarily yielded phenotypically homogeneous groups. As shown in Table 4, the two major clusters, groups 1 and 2, give variable reactions for nitrate reduction, maximum growth temperature, and, especially, sugar fermentation tests.

The strains studied by Gordon et al. (18) all produced acetylmethylcarbinol, grew in the presence of 0.02% azide, acidified litmus milk (one exception), and hydrolyzed starch. These workers appear to have fortuitously selected only group 1 organisms for their study. Even among those strains variable reactions were observed for the fermentation of

TABLE 5. Characterization of group 1 and 2 strains with the API 20E and API 50 CHB test systems

Characteristic ^a	% of strains showing positive reaction	
	Group 1 (n = 9)	Group 2 (n = 22)
API 20E tests		
ONPG	30	0
ADH	0	0
LDC	0	0
ODC	0	0
Citrate (Simmons)	0	0
H ₂ S	0	0
Urease	0	0
TDA	0	0
Indole	0	0
Voges-Proskauer	100	36
Gelatin	0	0
Nitrate	0	0
API 50CHB tests		
Glycerol	100	64
Erythritol	11	14
D-Arabinose	22	0
L-Arabinose	11	27
Ribose	44	77
D-Xylose	33	99
L-Xylose	0	0
Adonitol	0	0
β-Methylxyloside	0	0
D-Galactose	100	9
D-Glucose	100	100
D-Fructose	100	100
D-Mannose	100	27
L-Sorbose	0	0
Rhamnose	22	18
Dulcitol	0	0
Inositol	22	9
Mannitol	0	82
Sorbitol	22	18
α-Methyl-D-mannoside	0	0
α-Methyl-D-glucoside	22	5
N-Acetylglucosamine	100	0
Amygdalin	33	0
Arbutin	33	0
Esculin	89	63
Salicin	44	18
Cellobiose	55	5
Maltose	100	87
Lactose	67	0
Melibiose	100	0
Sucrose	99	5
Trehalose	100	100
Inulin	0	0
Melezitose	0	0
D-Raffinose	67	0
Starch	78	0
Glycogen	0	9
Xylitol	0	5
β-Gentiobiose	44	0
D-Turanose	33	0
D-Lyxose	0	0
D-Tagatose	0	0
D-Fucose	0	0
L-Fucose	0	0
D-Arabitol	22	0
L-Arabitol	0	0
Gluconate	22	0
2-Ketogluconate	0	0
5-Ketogluconate	0	0

^a ONPG, o-Nitrophenyl-β-galactosidase; ADH, arginine dihydrolase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; TDA, tryptophan deaminase.

arabinose, mannitol, and xylose; for the utilization of citrate; for the reduction of nitrate to nitrite; and for the hydrolysis of casein. Thus, for both *B. coagulans* sensu stricto and group 2 strains, certain characteristics are inherently variable.

Because group 2 constitutes a major portion of the *B. coagulans* sensu lato group, efforts have been made to determine its taxonomic position. As shown in Table 3, group 2 is not closely related genetically to organisms that have G+C contents of 37 to 44 mol% or grow at 50°C or both (namely, *B. alcalophilus*, *B. azotoformans*, *B. badius*, *B. firmus*, *B. globisporus*, *B. laterosporus*, *B. macquariensis*, *B. marinus*, *B. megaterium*, *B. pumilus*, and *B. subtilis*). In addition to their inability to grow at 60°C, these organisms are distinguishable from group 2 strains by the key characteristics listed in Table 6.

Group 2 organisms are also phenotypically distinct from previously described thermophilic *Bacillus* spp. (Table 7). In contrast to group 2, the known thermophiles are generally strictly aerobic, have G+C contents ranging from 45 to 68 mol%, and do not grow at 25°C. Furthermore, the lowering of the pH in Voges-Proskauer broth to 5.0 or less and hydrolysis of casein differentiate *B. stearothermophilus*, *Bacillus thermoglucosidasius*, and *B. brevis* from group 2. *B. stearothermophilus* and *B. thermoglucosidasius* are further distinguished by their inability to grow at pH 5.7 and their ability to hydrolyze gelatin. In contrast to group 2 strains, *Bacillus schlegelii* and *Bacillus tusciae* are facultatively chemolithoautotrophic and reduce nitrate to nitrite. The acidophilic nature of *Bacillus acidocaldarius* further differentiates it from the group 2 organisms.

On the basis of the data, we consider group 2 organisms to be strains of a new species, for which we propose the name *Bacillus smithii*. A description of this species is given below.

***Bacillus smithii* sp. nov.** *Bacillus smithii* (smi'thi.i.L. gen. n. smithii named after Nathan R. Smith, the American bacteriologist who has made fundamental contributions to the taxonomy of *Bacillus*) vegetative cells are rods that measure 0.8 to 1.0 by 5.0 to 6.0 μm and are motile by peritrichous flagellation. Oval or cylindrical endospores (0.6

TABLE 6. Key characteristics that differentiate currently recognized *Bacillus* spp. from group 2

Species	Key characteristic(s)	Reference
<i>B. alcalophilus</i>	Grows at pH 10	50
<i>B. azotoformans</i>	Produces N ₂ from nitrate	36
<i>B. badius</i>	Does not ferment glucose	18
<i>B. firmus</i>	Does not grow anaerobically or at 50°C	18
<i>B. globisporus</i>	Forms round spores; low optimum growth temperature of 20°C	18
<i>B. laterosporus</i>	Unusual spore morphology; does not grow at pH 5.7	18
<i>B. macquariensis</i>	Low optimum growth temperature of 20°C	18
<i>B. marinus</i>	Low optimum growth temperature of 20°C	18
<i>B. megaterium</i>	Does not grow anaerobically or at 50°C large cells	18
<i>B. pumilus</i>	Does not grow anaerobically or at 60°C; produces acetylmethylcarbinol	18
<i>B. subtilis</i>	Does not grow anaerobically or at 60°C; produces acetylmethylcarbinol	18

TABLE 7. Differentiation of *B. smithii* from other thermophilic *Bacillus* species

Characteristic	<i>B. smithii</i>	<i>B. stearo-thermophilus</i> ^a	<i>B. thermo-glucosidarius</i> ^b	<i>B. brevis</i> ^{a,a}	<i>B. acido-caldarius</i> ^d	<i>B. schlegelii</i> ^e	<i>B. tusciae</i> ^f
Growth in:							
Nutrient broth at pH 6.8	+	+		+	—	+	—
Nutrient broth at pH 4.5	—	—	—	ND	+	—	—
Sabouraud broth at pH 5.7	+	—	—	d	d	—	ND
Growth at:							
70°C	—	d	—	—	d	+	—
65°C	d	+	+	—	+	+	—
60°C	+	+	+	+	+	+	—
25°C	+	—	—	d	—	—	—
Growth with 0.02% azide	—	—	—	—	ND	ND	ND
Anaerobic growth	+	—	—	—	—	—	—
Autotrophic growth with H ₂ + CO ₂	—	ND ^h	ND ^h	ND ^h	—	+	+
Voges-Proskauer reaction	—	—	—	—	—	—	ND
pH in Voges-Proskauer broth							
5.0	+	—	—	—	ND	ND	ND
7.0	—	—	—	+	ND	ND	ND
Nitrate reduction to nitrite	—	d	+	—	ND	+	+
Hydrolysis of:							
Casein	—	d	+	+	ND	—	ND
Starch	d (weak)	+	+	—	+	—	—
Gelatin	—	+	+	ND	ND	—	ND
Litmus milk							
Coagulated	—	—	—	—	ND	ND	ND
Acidified	—	—	—	—	ND	ND	ND
G+C content (mol%)	38.7–39.7	44–53 ⁱ	45–46	46 ^j	60–62	64–68	57–58

^a Data from reference 18.^b Data from reference 48.^c Data only for thermophilic strains of the species according to Gordon et al. (18).^d Data from reference 12.^e Data from reference 41.^f Data from reference 4.^g +, Positive for ≥90% of the strains; —, negative for ≥90% of the strains; d, positive for 11 to 89% of the strains; ND, not done.^h Strains of these species growing autotrophically with H₂ + CO₂ have not been isolated.ⁱ The G+C content of the type strain is 51.9 mol% (16).^j Data from reference 16.

to 0.8 by 1.3 to 1.5 µm) are produced terminally or subterminally in nonswollen sporangia or in some cases slightly swollen sporangia. The cell walls of vegetative cells contain diaminopimelic acid. The main menaquinone is MK-7; also present is MK-6. Agar colonies are nonpigmented, translucent, thin, smooth, circular, and entire and measure about 2 mm in diameter.

Chemoorganotrophic. Facultative thermophile. Grows at 25 to 60°; most strains grow at 65°C. Facultatively anaerobic. Grows at pH 5.7; does not grow at pH 4.5 or 7.7 in nutrient broth. Does not grow in the presence of 3% NaCl, 0.001% lysozyme, or 0.02% azide (except two strains). Acetyl-methylcarbinol, H₂S, and indole are not produced. In Voges-Proskauer broth, the pH ranges between 4.3 and 4.7.

Catalase and oxidase are produced. Nitrate is not reduced to nitrite. Casein, chitin, egg yolk lecithin, and gelatin are not hydrolyzed. Starch is weakly hydrolyzed. Hydrolysis of pullulan and esculin is variable. DNA and hippurate are hydrolyzed. *o*-Nitrophenyl-β-galactosidase is not produced.

Utilization of citrate and propionate is variable. Acetate, fumarate, malate, and succinate are utilized.

Arginine dihydrolase, lysine and ornithine decarboxylases, phenylalanine and tryptophan deaminases, and urease are not produced. Tyrosine is not decomposed.

Litmus milk is not coagulated or acidified, but is usually alkalized.

Acid but no gas is produced from D-fructose, D-glucose,

and trehalose. Acid production from L-arabinose, erythritol, D-galactose, glycerol, maltose, mannitol, D-mannose, D-ribose, L-rhamnose, salicin, sorbitol, and D-xylose is variable. A small proportion (10% or less) of the strains metabolize the following substrates: *N*-acetylglucosamine, adonitol, amygdalin, D-arabinose, D-arabitol, arbutin, cellobiose, dulcitol, D- and L-fucose, β-gentiobiose, gluconate, glycogen, inositol, inulin, 2- and 5-ketogluconate, lactose, D-lyxose, melezitose, melibiose, α-methyl-D-glucoside, β-methyl-xyloside, α-methyl-D-mannoside, raffinose, L-sorbose, starch, sucrose, D-tagatose, D-turanose, and xylitol.

The DNA buoyant density range for 21 strains was 1.6917 to 1.6940 g/cm³, and the G+C contents determined from these values ranged from 38.1 to 40.4 mol%. As determined by the thermal melting point method the G+C contents ranged from 38.7 to 39.7 mol% (five strains tested).

Isolated from evaporated milk, canned foods, cheese, and sugar beet juice from extraction installations.

The type strain is NRS-173, which has been deposited as strain NRRL NRS-173 in the Agricultural Research Service Culture Collection, Peoria, Ill.

Description of the type strain. The type strain has the characteristics of the species; with respect to the variable characteristics, it ferments L-arabinose, D-galactose, maltose, mannitol, glycogen, D-ribose, and L-rhamnose. Citrate and propionate are utilized. The G+C content is 40.2 mol%. This strain was isolated from cheese.

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